FURTHER EXPERIMENTS IN CROSS- AND SELF-FERTILIZATION OF CIONA AT WOODS HOLE AND CORONA DEL MAR

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Of the many studies that have been carried out on the development of the eggs of marine animals, it is surprising how little attention has been paid to the development of 100 per cent normal embryos or larvae. Even in the case of the sea-urchin, where hundreds of experiments have been reported, the percentage of normal embryos is seldom, if ever, recorded. Many workers are contented with batches of these eggs in which the fertilization membrane is given off in practically all the eggs, but even then the number of such eggs that produce normal plutei is seldom stated. E. E. Just, almost alone, has emphasized the importance of using normal sea-urchin's eggs, and also the need of paying the strictest attention to the environment in which the eggs develop. Albert Tyler has also taken great care to use only normally developing eggs in his physiological experiments.

Sea-urchins brought back from the collecting grounds in crowded jars or in buckets to the laboratory or those kept in floating cars, or kept without food in aquaria are sometimes recognized as a source of abnormalities, but the condition of their eggs is, as a rule, ignored.

In the earlier work on artificial fertilization the occurrence of " swimmers " was often reported as indicating a successful result, but every embryologist knows that " swimmers " are abnormal embryos. Only as methods improved were normal plutei reported, but practically never was the percentage of normals given.

Most of those who have used sea-urchins are familiar with the fact that individuals are frequently met with whose eggs fail to give off a normal fertilization membrane, although the eggs appear to be normal. To what extent this is due to the eggs not being " ripe " or overripe is seldom known, even though in sea-urchins the polar bodies have been given off when the eggs leave the wall of the ovary and are presumably mature. In the starfish, on the contrary, the eggs free in the ovary still contain the large germinal vesicle which will disappear to form the polar spindle when the eggs are removed to sea water. It is well known, however, that such eggs often develop abnormally when fertilized. When a starfish spawns normally, the eggs, before extrusion, have matured, i.e., the germinal vesicle has disappeared, and it is recognized that such eggs give, as a rule, normal development. Of course, it is known that polyspermy in the sea-urchin is one of the factors of abnormal development, and this holds for other animals whose eggs are fertilized in the laboratory. Fortunately, in *Ciona* polyspermy is a relatively rare occurrence, and this source of abnormal development is practically eliminated. Nevertheless, sets of eggs that give 100 per cent two-cell stages sometimes give rise to some or to many abnormal embryos.

So far I have referred to what appear to be internal factors in the eggs themselves which cause abnormal development. What these factors are, aside from immaturity or over-ripeness, is generally unknown and has been little studied. On the other hand, it is well known that developing marine embryos are extremely sensitive to external factors such as temperature, salinity, impurities in the water, bacteria, etc. In forms that develop slowly (outside the parent), the chances of unfavorable conditions appearing are much increased, but even in rapidly developing forms, such as *Ciona,* external factors may also play a significant rôle. Fortunately, in this animal the completely formed tadpoles develop at 22° C. in fifteen hours or less, and it is not difficult to control the environmental factors during this time. But, as the following experiments clearly show, unless the eggs are thoroughly washed and the excess of sperm removed, abnormal development is apt to occur. Even then, however, different individuals may give quite different results when the environment is apparently the same for all. Perhaps this may be expressed by saying that the eggs of different individuals respond differently to the same environmental differences. If this is the correct interpretation, as the evidence at hand seems to indicate, it is evident that contradictions may appear when the eggs of different individuals are treated in the same way. This possibility makes the problem difficult, but repetitions of the same kinds of experiments have helped to clear away some of the apparent contradictions.

The most puzzling problem is the occurrence of both normal and abnormal larvae in the same culture of *Ciona.* The numbers may vary from one or two normals to 99.5 per cent normals. Sets of eggs of one individual fertilized by sperm of one other individual tend to give the same proportions, under external conditions that are the same, but even here, exceptionally, the ratios may vary. Different samples of eggs may account in part for these differences, even when an attempt is made to make the samples the same. Differences in the position of the eggs in the dishes may make for differences, but when few are present they space themselves equally on the bottom, and even when several thousand are present in a thin layer of water, practically 100 per cent may be normal. "Accidents" of development, such as the position of the cleavage planes, may be another factor, but it is impossible to say what causes these accidents,—whether they are internal or external.

The western *Ciona,* like the eastern one, sets free its eggs and sperm in the early morning in response to a change from darkness to light. In general, therefore, fewer eggs are found in the oviduct in the later morning than in the afternoon, and the former may be said to be younger than the latter, although all have formed the polar spindle before leaving the ovary. No difference has been observed between the younger and older eggs in respect to the development of normal embryos. When Cionas are brought into the laboratory and kept in running water, or in aerated water, the eggs may accumulate, in some individuals at least, until the oviduct is swollen with them. They may be two or three days old, yet produce as many normals as eggs from freshly-caught individuals. If the water is changed daily and kept clean, and only a few Cionas kept in the same jar, the eggs are good for at least three days.

My interest in the problem of abnormal development in *Ciona* is only secondarily concerned with the problems mentioned above. It became necessary to find out to what extent the abnormal development is due to internal factors on account of its possible relation to the genetic problem of self-sterility and cross-fertilization in *Ciona.* If abnormal development is due to inherited genetic factors, then its occurrence, if regular and internal, might be due to genetic lethals whose presence might bear on the main problem.

New Experiments with Ciona at Woods Hole, Mass.

The results of experiments with *Ciona* at Woods Hole which I carried out during the years 1904 and 1910, are in some instances more erratic or irregular than those obtained in recent years with what is said to be the same species on the California coast. This is true, moreover, of experiments that I carried out in 1904 at Coronado Beach, California, on *Ciona* which is undoubtedly the same type that is found at Corona del Mar. I was inclined to think that the methods of handling the eggs in the earlier experiments at Woods Hole might account for the differences, and therefore when I had a chance to test out the Woods Hole form during September, 1940 I carried out some experiments that I hoped would show whether the differences are due to the earlier technique employed, or to differences in the material itself. Two kinds of tests were carried out. There were five of the 5 × 5 cross- and self-experiments, and five experiments of a different kind.

In the 5 × 5 tests a larger amount of water was used than in the former experiments at Woods Hole; also the eggs were washed in one or two changes of water. The eggs were then concentrated in a small amount of the water, and 7 to 10 drops of the eggs were then transferred to Stender dishes containing 20 cc. sea water. The cleavages were noted, and after 20 to 24 hours the kind of development recorded. In all cases (100 in all) the cross-fertilized eggs gave 100 per cent cleavage. There were no failures to cross-fertilize. In the 25 selfed lots most gave no cleavage (except that in one case 95 per cent cleaved), but there were a few cleavages in some lots. It is to be noted that only 5 drops of sperm suspension were used, and the concentration of sperm was not large since the individuals were small.

The first lots came from a float in the seal-pool of the Fish Commission. There were at least a thousand Cionas on the float when removed; most of them were only half grown, but a few had eggs and sperm. Those reported in the first three sets came from this float; those in the two remaining sets came from the supply tank on the roof of the laboratory. These were also small individuals and only a few were mature or had enough eggs for the tests.

I. (Sept. 16, 1940.) All of the cross-fertilized eggs gave 99 or 100 per cent cleavage (except one that gave 50 per cent). The five selfed sets gave 0, 25, 25, 50, 40 per cent cleavage. The total number of eggs in the dishes (20 cc.) was between 50 and 100. After 24 hours it was found that three lots of eggs, viz., *c, d, e,* gave very abnormal embryos; one lot, *b,* gave all normal or late abnormal stages; and another lot, *a,* gave normal, late abnormal tadpoles, and early abnormal tadpoles. It was very noticeable that the eggs rather than the sperm determined the kind of devlopment that took place. Over and beyond this, however, there are differences in the different lots of eggs of the same set that seem to follow the sperm, or the combination of eggs and sperm.

II. (Sept. 16, 1940.) These Cionas came from the same float as the last. They had been kept in the laboratory in running water for one day. The 20 crossed lots gave 100 per cent normal 2-cell stages; rarely one or two of the selfed lots divided. Five drops of sperm suspension were used, and 15 cc. sea water. Taking the crossed eggs in the horizontal lines, *aB* and *aC* gave abnormal embryos, *aD* normal tadpoles and *aE* late embryos. The next lot, *b* eggs, gave all normal tadpoles as did the *c* eggs. The *e* and the *d* eggs gave nearly all abnormal embryos. The eggs appear responsible for the differences, and not the sperm, which was the same in each of the vertical lots.

III. (Sept. 18, 1940.) These came from the same source as I, but had been kept in running water in the laboratory for two days. Only a few of the Cionas had enough eggs for the experiment. The sperm duct was better. Five drops of sperm suspension were used, and the eggs were in 16 cc. sea water when fertilized. Most of the supernatant fluid was then drawn off the eggs, and 10 drops of eggs added to 20 cc. sea water in Stender dishes. There were 75 to 150 eggs in each dish (fewer in *d*). All 20 crosses gave 100 per cent regular cleavages, except *Ad* and *Bd,* that had a few irregular cleavages. None of the eggs selfed. The cross-fertilized eggs gave abnormal embryos that died young (stage *d* and *e*). (*See* Morgan, 1938*a*, p. 305.)

IV. (Sept. 19, 1940.) These Cionas came from the tank on the roof of the laboratory. The cleavage was not observed. The eggs were washed twice in sea water and 12 drops transferred to each dish (20 cc.). Five drops of sperm suspension were added to each. Four of the egg-lots gave normal and nearly normal tadpoles, one gave very abnormal embryos. Two dishes, *eA* and *eB,* contained only 10 cc. sea water and these gave very abnormal embryos, although the other two, *eC* and *eD,* gave normal and abnormal tadpoles.

V. (Sept. 21, 1940.) This lot also came from the tank and had been kept two days. The eggs were washed once; then eight drops were added to each Stender containing 20 cc. sea water, where they were fertilized (5 drops). Four of the lots had some normal and bent tadpoles, but even these dishes had, for the most part, abnormal embryos; one lot had only very abnormal tadpoles. It is not evident in this set whether the great variability in each dish and between different dishes is due to the condition of the eggs or to the presence of the sperm in the dishes. One dish of selfed, *eE,* gave 113 short abnormal tadpoles, 12 late abnormal and 4 abnormal embryos; the other selfed lots had nearly all unfertilized eggs.

It is quite evident even from these few experiments that practically 100 per cent cleavage occurs if the eggs are washed and not too much sperm added. Evidently these Cionas at Woods Hole behave in the same way as those at Corona del Mar. The cleavage irregularities in the 1904 and 1906 experiments must have been due to handling. It is also clear here that despite the occurrence of the normal 2-cell stage, the eggs of several of the individuals gave abnormal embryos and abnormal tadpoles.

Some further tests were made on some of these Woods Hole Cionas in order to study the effect of external conditions on development.

(Sept. 19, 1940.) The eggs were washed, then cross-fertilized (*A* by *b* and *B* by *a*) by 5 drops of sperm suspension. All eggs in both sets cleaved. In the two-cell stage the eggs (5 drops) were transferred to 10, 20, 40 cc. of sea water. All sets gave abnormal embryos. These results are clearly due to the condition of the eggs and are nearly the same as two of the lots in the 5 by 5 tests of the same date. The other three lots of the same 5 by 5 test gave mostly normal tadpoles.

(Sept. 21, 1940.) Two small Cionas from the tank were used. The eggs were washed once, and 10 drops were transferred to 10 and 20 cc. sea water, where they were fertilized (5 drops). *A* by *b*, in 10 cc., gave late abnormal embryos, and in 20 cc. 49 nearly normal tadpoles and 19 normals. *B* by *a*, in 10 cc., gave the same result as above; and in 20 cc. there were 85 normals and 3 abnormal tadpoles. Here 20 cc. gave distinctly better results than 10 cc. It will be noted that 5 drops of the sperm suspension were left in each dish.

(Sept. 21, 1940.) The water was changed once on the eggs, then they were cross-fertilized (5 drops). The supernatant water was then largely removed and 8 drops of eggs added to each dish of 10 and 20 cc. sea water. The *A* by *b* in 100 cc. sea water gave 95 per cent normals; and in 20 cc. gave 99.5. But *B* by *a* in 10 cc. gave 145 abnormal embryos and in 20 cc. gave only 9 normal tadpoles, 6 bent and 109 abnormal tadpoles. Here also the results were a little better when more water was present, but there was a striking contrast between the reciprocal crosses.

In a repetition of the same experiment on the same date, *A* by *b* eggs in 10 and in 20 cc. sea water gave abnormal tadpoles; *B* by *a* eggs in 10 cc. gave abnormals as before, but in 20 cc. gave 393 normals, 109 twisted tadpoles and 62 eggs or young embryos.

(Sept. 24, 1940.) The eggs were washed once. Ten drops of eggs were then transferred to 20 cc. sea water in two dishes (*a* and *a*′). Here they were fertilized with 5 drops of sperm suspension. After 15 minutes the water was removed from one dish (*a*′) and replaced by new sea water. All sets gave 100 per cent cleavage. The embryos in *a* died young, but *a*′ gave 6 nearly normal, 40 crooked tadpoles, and 76 very abnormal tadpoles. Evidently the latter set went further than the former (in which the sperm was left). The reciprocal cross, *B* by *a*, gave somewhat better results. In *b* there were 11 normal, 12 crooked, and 59 very abnormal tadpoles. In *b*′ there were 14 normal, 14 crooked and 49 very abnormal tadpoles. The results were the same in *b* and *b*′, and somewhat better than in *a* and *a*′.

It is very noticeable that with the better technique practically all the cross-fertilized eggs in the set of experiments gave 100 per cent cleavage, but nevertheless there was a high percentage of abnormal development. The latter can only be ascribed to internal conditions in the eggs or sperm, probably in the former. The Cionas were just reaching maturity, i.e., about half normal size. The test was transparent and thin. It seems safe then to ascribe the abnormal development to immaturity of the animals although the eggs had every appearance of being normal. There is here a rather sharp contrast between the normal behavior of the eggs and sperm with respect to cross- and self-fertilization, and the failure of many of the fertilized eggs to produce normal tadpoles.

Review of Earlier Work

In the summer of 1903 (see *Jour. Exper. Zool.,* 1904) I made four 5 by 5 experiments on Cionas at Woods Hole, Mass. In one of them practically all the cross-fertilized eggs segmented. In another the sperm completely failed in two cases and practically in a third (giving 30 per cent in one case), although the eggs were good as shown in the other two cases. In a third test the sperm was not very good (except in one case). In a fourth test the sperm was not good in one case (except with one set of eggs where it gave 75 per cent cleavage). It seems now evident that the failure to cross-fertilize was due to the sperm suspension, and not to the conditions under which the tests were made.

Six years later (1909. See Roux's Archiv., 1910) abundant material was available at Woods Hole and fifteen 4 by 4 tests were carried out (180 crosses in all). Of these only two gave uniformly good results. In some, the four lots of eggs were obviously poor, in others the sperm, but in all of them (except two) there were inconsistent results as though certain combinations were incompatible, but in the light of the results with the California type of *Ciona* it seems highly improbable that these failures to cross-fertilize (i.e., to cleave) were due to the presence of individuals with identical genetic composition, and there is no evidence that the results were due to differences in the environment.

In 1932 (Sept. 19) a few large Cionas were brought to me at Woods Hole. I tried out one 5 by 5 experiment for the cleavage. All cross-fertilized eggs gave nearly 100 per cent (one 95 per cent). The selfed lots gave 0, 0, 8, 0, 4 per cent. This is the same kind of result as with the California type. Some other eggs were treated with crab juice (*Callinectes*) for four hours, and then selfed. They gave practically 100 per cent cleavage. When treated with acid sea water and selfed they gave 100 per cent cleavage. In both respects these eggs agree with the California Cionas. It is noticeable, in comparison with the later (1940) experiments at Woods Hole on small immature Cionas, that these Cionas were large, and the results were uniform and good.

In the summer of 1904 I spent a couple of weeks at Coronado Beach, California, at the abandoned yacht club that had been used earlier as a marine station of the University of California. There were quantities of large Cionas on the float at the station. The laboratory room was very warm during the daytime and the glassware insufficient. Only small saltcellars were available for the eggs, etc. The results of eight 5 by 5 tests were published in 1905. On the whole, the results for cleavage were poor and irregular. There were at least 17 cases where the sperm gave no cross-fertilization or very little, and almost as many cases where the eggs were poor, in the sense that they did not cleave although they had every appearance of normal eggs. That the heat in the laboratory was not the cause of the failure to cleave was evident since in every set there were cases of normal (100 per cent) cleavage, but the heat may account for the almost entire failure to give normal embryos. The dishes were treated uniformly, although owing to the abundance of sperm, too much may have been used, which, while not affecting the cleavage, would affect the development. The only explanation I can offer is that the temperature of the water in the basin around the float had injured the reproductive cells before the Cionas were removed.

The Causes of Variability of Normal and Abnormal Development

From March to the end of June and again during October and January, 1940–41, I repeated, with improvements in the technique, some of the earlier experiments that had been made to find what conditions determine whether normal or abnormal development takes place, whether internal or external.

It had become evident from previous experiments that better, i.e., more uniform results, take place if the eggs are thoroughly washed, then fertilized with a few drops of sperm suspension, most of the supernatant fluid removed, fresh water added, and, after the eggs have settled, most of this water also removed leaving only enough to supply a drop of eggs to each of the dishes in which the eggs are to develop. An experiment of this kind was made (Oct. 19) at Corona del Mar with Cionas freshly brought in. The eggs were washed, and 10 cc. of fresh sea water added. They were cross-fertilized by 5 drops of sperm suspension, and after 10 minutes the supernatant fluid was drawn off (except 20 drops). One drop of these fertilized eggs was added to 10 cc. sea water (in each of 10 dishes). The eggs were brought back to Pasadena in closed Stender dishes, except the last four that were brought back in closed vials. *A* by *b* gave the following figures:

94, 97, 99, 93, 99, 99, 99, 99.5, 99.5, 99.5 per cent tadpoles

The reciprocals, *B* by *a,* gave a much smaller percentage of normals:

49, 46, 28, 24, 3, 59, 38, 31, 35 per cent tadpoles

The latter figures are less than half the former and there is more variability, although the external conditions were made as like as possible. The differences in the two cases seem to depend on the eggs or on the sperm (or both). The amount of sperm carried over with one drop of fertilized eggs must have been too small to affect the results, even if there was some initial difference in the sperm suspensions.

The next day (Oct. 20) the same experiment was carried out with fresh eggs from the adult Cionas that had been brought to Pasadena. The results were very uniform, giving 99 per cent throughout (except one, 95 per cent). Repeated four times (Oct. 20–21) all gave about 100 per cent normals.

In order to test further (Nov. 3) whether the transportation of segmented eggs in closed Stender dishes gives the same results as do those left at Corona del Mar, since in one case not reported here some of the former were abnormal, the eggs of fresh Cionas were washed twice, then fertilized, and after 15 minutes the supernatant fluid was taken off (except about 20 drops). Then one drop of these fertilized eggs was added to each of 20 Stender dishes (20 cc.). Ten of these dishes, *A* by *b*, were taken (after 4 hours) to Pasadena. The percentages of normal embryos were: 52, 74, 80, 95, 91, 52, 96, 0,[1] 55, 80 per cent; average 75.0 per cent. The other ten dishes left at the shore gave: 100, 99.5, 100, 99.5, 99.5, 0,[1] 60, 100, 70, 100 per cent; average 92.1 per cent. In each set there was one dish that gave no normals; both had dirt in them and were disregarded. Those left at the shore had a higher level of normals, which is probably not significant. Difference in temperature was not involved here, since the car was cool. Six dishes of the reciprocals, $B \times a$, were also tested in the same way. Those taken to Pasadena gave: 93, 87, 87, 93, 92 per cent. These average a little better than the corresponding *A* by *b*. Those left at Corona del Mar gave: 100, 99, 99, 99.5, 93 per cent. The differences are probably not significant since the percentages are estimates only.

The following experiment was made with Cionas (Nov. 11) that had been kept for two days (with change of water). The eggs were washed twice and all fertilized by five drops of sperm suspension. After 15 minutes the supernatant water was changed, and one drop of eggs put into each of ten Stender dishes containing 15 cc. sea water. The reciprocal test was treated in the same way. The percentages in the first dishes were: 68, 69, 69, 73, 79, 80, 70, 78, 76, 80 per cent. The abnormals were of two kinds, viz., late abnormal tadpoles and early abnormal embryos in about equal numbers. The reciprocal ten dishes gave: 96, 97, 97, 96, 96, 98, 97, 99, 97, 99 per cent. The percentages

[1] All were late abnormal tadpoles. The contrast with the others is not so great as it appears to be.

are higher here than in the other ten dishes, and there is less variation. Many of the eggs (4224, and 4624) were left over in the original Syracuse dishes containing little water. Despite the small amount of water and the large number of eggs, the percentages (A by $b = 80$ per cent, B by $a = 98$ per cent) were not very different from those above.

The Cionas used in the next experiment had been brought to Pasadena (Oct. 12). Two days later (Oct. 14) the eggs of one of them were distributed in 10 dishes (10 cc. sea water), fertilized by sperm of another individual (one drop). The sperm was left with the eggs in this case, but only one drop to each dish. The actual counts were:

Normal Tadpoles	163	138	196	188	65	43	89	104	108	80
Abnormal tadpoles:	10	2	2	2	4	1	1	—	1	—
Abnormal embryos:	99	112	137	157	55	86	87	102	71	75

Counting both kinds of abnormals together, the percentages of normals are 60, 55, 58, 54, 52, 33, 50, 50, 60, 52. The variability is not much, but the percentages of normals are low.

The reverse (not reciprocal) test, with eggs that came from the same individual that supplied the sperm above, fertilized by sperm from one other *Ciona,* was much the same, but somewhat better:

Normal Tadpoles	45	78	50	65	102	70	100	124	59	165
Abnormal tadpoles:	29	12	12	2	8	29	11	13	30	16
Abnormal embryos:	15	13	8	8	21	22	18	22	16	30

The percentages are 50, 75, 71, 87, 78, 58, 78, 78, 56, 78. The percentages of normals are low in both these tests, and not obviously environmental.

Since the experiments in which reciprocals are tested often show differences in the proportions of normal embryos, some further tests (Jan. 10 and 14) were made in which the eggs were first washed in two changes of water, and, after fertilization, the supernatant fluid was changed twice. At the two-cell stage one drop of eggs was added either to 10 cc. in a Syracuse dish or to 20 cc. in a Stender. The dishes had been washed with a weak hydrochloric acid solution, put into running tap water for two hours and then rinsed in distilled water. In one case (10 cc.) A by b gave 100 per cent; B by a 80 per cent. In another case (10 cc.) A by b gave 90 per cent and B by a 80 per cent. There were two dishes of each which gave the same results. Also the many left-over eggs in each case gave approximately the same ratios. Furthermore, some of the unfertilized eggs kept in another dish and fertilized by sperm of a third individual gave about the same kind of results. The same statement may be made for two other reciprocal pairs in 20 cc. sea water. These results are in line with previously recorded cases showing often different proportions of normal tadpoles in reciprocals.

Finally another test (Jan. 13) of the same kind with the same preliminary precautions was made in which ten similar dishes (20 cc.) were made up of the cross and ten of its reciprocal. This experiment was made as a further check on different dishes of the same sort which should give more precise confirmation as to the reliability of the experiments. The actual counts are given in Table I. In the first column the number of normal tadpoles is given; in the second the number of abnormal tadpoles which were crooked or twisted; and in the third the abnormal embryos that had died at an early stage without evident differentiation.[2]

Table I

	A by *b*					*B* by *a*			
	Normal	Abnormal Tadpoles	Abnormal Embryos	Percentage		Normal	Abnormal Tadpoles	Abnormal Embryos	Percentage
1	247	74	12	74	1	54	23	7	64
2	138	2	11	91	2	22	0	4	85
3	275	17	11	90	3	17	8	2	63
4	137	11	11	86	4	40	7	1	83
5	167	0	9	95	5	88	0	9	91
6	184	5	7	94	6	28	0	1	96
7	332	8	17	93	7	32	0	1	97
8	63	102	4	37	8	1	205	9	0.5
9	111	4	8	90	9	96	0	3	97
10	53	111	11	30	10	87	0	9	90

Several of the ratios in Table I call for comment. The ratios are made up of the normals against the two kinds of abnormals taken together. Except for 8 and 10 in *A* by *b*, where the ratios are 37 and 30 per cent, the other ratios are nearly the same. In these two the low ratio is due to an excess of "abnormal tadpoles." Again, in *B* by *a* the ratios are nearly the same, except for 1, 3 and 8. Here also the low ratios are due to excess of abnormal tadpoles and not to younger stages, i.e. "abnormal embryos." Number 8 had only one normal tadpole. The total average for *A* by *b* is 77.2 and for *B* by *a* 76.9. If number 8 is eliminated, the difference between the two crosses is small. I am in-

[2] The left-over eggs (about 2004) of *A* by *b* gave about 99.5 per cent normal and 5 per cent early embryos, and *B* by *a* (about 700 eggs) gave about 80 per cent normals, 15 per cent abnormal tadpoles and 5 per cent abnormal embryos. These results are about the same as those in Table I, where fewer eggs and much more water were present. In addition a few (150 and 100) eggs of *a* were fertilized each by sperm from a third individual, and gave, respectively, *C* by *a* about 95 per cent normal and *C* by *b* 95 per cent normals, which are as good percentages as the best in the table.

clined to think that the "abnormal embryos" are due to internal factors. Polyspermy is rare and only a part of this kind of embryo can be assigned to it; the others are probably due to failure to cleave normally in an early stage. On the other hand, most of the "abnormal tadpoles" seem to be due to environmental factors, especially the extreme cases. The individuals of this group have for the most part developed nearly normally to a late stage and their defects are due to failure to straighten out at the time of hatching. Nevertheless, since in the less extreme cases of low ratios the large majority of the eggs have developed normally, the environmental factors must have been nearly normal, which would mean that some of the eggs were more sensitive to outside agents than are others. This conclusion is borne out in some of the earlier experiments where more normals were present when the eggs developed in a larger volume of sea water, which would tend to dilute those external agents that act injuriously. In the earlier experiments, where reciprocal crosses were made, differences in the sperm suspension was probably one of the factors that would account for the difference between the cross and its reciprocal, but in these later experiments that factor is practically eliminated by washing the eggs and removing the supernatant sperm suspension. The eggs were thoroughly mixed and one drop only of the eggs was added to each 10 or 20 cc. of sea water. It is unlikely then that the differences in the dishes of the same composition are due to factors of this kind, and can only be ascribed to differences in the dishes themselves. A number of further tests of reciprocals were made which need not be recorded here, but which confirm the conclusion that reciprocals may consistently give different ratios which can only be due to internal factors in the eggs. Since no definite ratios appear in the data, it is highly probable that there is no specific lethal genetic factor involved.

The Development of Eggs from Different Levels of the Oviduct

In order to find out whether eggs from different regions of the oviduct give different percentages of normals, the following experiments were made. A very large *Ciona,* freshly collected (Nov. 23, 1940), was opened and the long oviduct was tied off by two ligatures into three sections, one near the outlet, one in the middle, and one next to the ovary. The first section would be expected to contain the oldest eggs, and the section next the ovary the eggs most recently set free from the ovary. Each section was opened separately. The eggs were washed twice, cross-fertilized with five drops of sperm suspension, which was largely removed when the eggs had reached the two-cell stage. Fresh water was added and then drawn off and replaced by fresh water (10 cc.). The first and second sections gave 100 per cent normal tadpoles. There were about 2,000 eggs in each dish. The third section (nearest the ovary) gave 85 per cent normals and 15 per cent bent and coiled tadpoles; 800 eggs in all. These differences are not significant as later results showed.

The experiment was repeated the next day (Nov. 24, 1940) on Cionas brought to Pasadena. The first and second (bracketed) are reciprocals as are also the third and fourth.

Section 1	Section 2	Section 3
{80	90	99
{99	70	70
{99.5	100	100
{99.9	100	100

There were from 800 to 1200 eggs in a dish (Syracuse) in only 10 cc. water, yet the percentage of normals was very high. There is no apparent difference in the percentages from the three different regions. It follows that different percentages of normals and abnormals in other experiments, when the eggs from the oviduct were used, are not due to age differences in the oviducal eggs.

The Cleanliness of the Glassware

The failure to get uniform results relating to normal development when the conditions of the experiments have been made as uniform as possible drew attention finally to the cleanliness of the glassware. As a rule the dishes were used for no other purpose, and were washed in tap water after each experiment, and drained, partly inverted, and not used again until dry. It was noticed that whenever drops of water had remained sticking to the glass and evaporated there a slight residue or stain was left. There was not sufficient reason to suppose such a minute amount of salts could affect the results, but, as a check, the dishes in one test were put into cleaning fluid, washed in running tap water and rinsed in distilled water, and used at once (Dec. 1). In four sets over 95 per cent normal tadpoles developed, but in one set the tadpoles failed to come out of the membrane. Clearly all the cleaning fluid had not been removed in this set. As a check the experiment was repeated (Dec. 2), omitting the cleaning fluid, and washing with distilled water and the double distilled water used in the last experiment. Normal tadpoles developed, 95 to 100 per cent. Finally, 10 dishes were scrubbed with trisodium phosphate, washed thoroughly in running water and drained. Washed eggs in the two-cell stage were added to 10 cc. sea water in Stender dishes. All gave late abnormal embryos. There were eight control dishes washed only in tap water which gave normals (most of them 100 per cent). When the same kind of experiment was repeated, but, after scrubbing the dishes in trisodium phosphate, they were washed, rinsed in 10 per cent HCl, and washed again, they gave 90 to 100 per cent normals, as did the controls washed in tap water, as also did some other dishes rinsed in HCl, and washed in tap water. As a further check other dishes were scrubbed in the trisodium phosphate and very thoroughly washed in running tap water. These also gave 99 to 100 per cent normals. It is evident from these experiments that the developing embryos are very sensitive to even traces of the chemicals used here in cleaning the dishes. The ordinary procedure of washing in tap water gives the best results, as a rule. Any salts that happen to remain as stains do not interfere with normal development. In fact, unless the utmost care is taken in removing the chemical material used to clean the dishes, there is more risk of causing abnormal development than from ordinary tap water.

Summary

A repetition of some of the earlier work on Cionas at Woods Hole (in 1904, 1905, 1910), with improvements in the technique that the later work at Corona del Mar had shown to be important, has made it clear, so far as cross-fertility and self-sterility are involved, that the Woods Hole type gives the same results as the California type. The very eccentric results concerning normal and abnormal development shown in the earlier experiments appeared again, and were found not to be due entirely to differences in technique, but to differences in the eggs of the Cionas themselves, connected, in part at least, with immaturity of the animals, even when their eggs and sperm appeared to be normal, and when the eggs cleaved normally, at least into two cells.

Some of the later work, carried out at Corona del Mar in 1940 when greater care was taken in removing the egg-water (by washing the eggs) and also in removing the excess of the sperm suspension, is reported. In most cases the eggs were fertilized en masse, and, after washing again and removing the excess of water, a drop or two only of the eggs was added to the sea water (10 to 20 cc.) in Stender dishes. The reciprocal cross was made in the same way. It was found that there is a marked tendency for all the dishes of the same cross to give closely the same percentages of normal tadpoles, but there were occasional dishes that gave more extreme variations (usually more abnormals). These are due to environmental factors. The cross, when compared with its reciprocal, frequently gives different percentages of normal development when the external conditions (water and dishes) are as nearly the same as possible.

Evidently, then, the eggs of different individuals in reciprocal crosses may give different percentages, although from a genetic (chromosomal) point of view the two are, on the average, identical *after fertilization.* It seems to follow, then, that this difference must lie in the cytoplasm of the eggs which in each case has been formed under the diploid condition of the eggs. There is also other evidence supporting such an interpretation. It should be pointed out, however, that, as a rule, the percentages of the two reciprocals are the same or nearly so. When they differ more markedly there are no definite ratios between them, so far as the observations go.

A number of experiments were also made to test whether differences in the dishes, used in the experiments after washing in tap water and drying, are responsible for the variability sometimes found in the same series. There is no evidence that this is the case if the dishes have been carefully washed and drained. On the other hand, if they have been cleaned by the ordinary chemical treatments there is evidence of effects on the development unless great care is taken to remove every trace of the cleaning fluid.

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